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PCT DK96 00231

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

HOFMAN BANG & BOUTARD, LEHMANN & REE A S
Hans Bekkevolds Allé 7
DK-2900 Hellerup
DANEMARK

Date of mailing (day month year)	
20 November 1997 (20.11.97)	
Applicant's or agent's file reference	**IMPORTANT NOTIFICATION**
2950538 WO	
International application No.	**International filing date** (day month year)
PCT/DK96/00231	31 May 1996 (31.05.96)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address	State of Nationality	State of Residence
MOURITSEN & ELSNER A S Lersø Parkallé 40 DK-2100 Copenhagen Ø DENMARK	DK	DK
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [X] the name [X] the address [ ] the nationality [ ] the residence

Name and Address	State of Nationality	State of Residence
M&E BIOTECH A S Kogle Allé 6 DK-2970 Hørsholm DENMARK	DK	DK
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[ ] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer Marie-José Devillard
Facsimile No. 41-22-740 14 35	Telephone No. 41-22-338 83 38

Form PCT/IB/306 (March 1994)

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ETATS-UNIS D AMERIQUE

in its capacity as elected Office

Date of mailing (day month year) 12 February 1997 (12.02.97)	
International application No PCT/DK96/00231	**Applicant's or agent's file reference** 2950538 WO
International filing date (day month year) 31 May 1996 (31.05.96)	**Priority date** (day month year) 02 June 1995 (02.06.95)
Applicant JENSEN, Martin, Roland et al	

1. The designated Office is hereby notified of its election made

[X] in the demand filed with the International Preliminary Examining Authority on:

23 December 1996 (23.12.96)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer:

Ingrid Hours

Telephone No.: (41-22) 730.91.11

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

HOFMAN-BANG & BOUTARD, LEHMANN & REE A S
Hans Bekkevolds Allé 7
DK 2900 Hellerup
DANEMARK

Date of mailing (day month year)
12 February 1997 (12.02.97)

Applicant's or agent's file reference
2950538 WO

IMPORTANT NOTIFICATION

International application No.
PCT/DK96/00231

International filing date (day month year)
31 May 1996 (31.05.96)

1. The following indications appeared on record concerning:

[ ] the applicant [ ] the inventor [X] the agent [ ] the common representative

Name and Address
HOFMAN-BANG & BOUTARD, LEHMANN & REE A/S
Adelgade 15
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State of Nationality:
State of Residence:
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
HOFMAN-BANG & BOUTARD, LEHMANN & REE A/S
Hans Bekkevolds Allé 7
DK-2900 Hellerup
Denmark

State of Nationality:
State of Residence:
Telephone No.:
Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:
The new address of the agent on the demand (Form PCT IPEA 401) has been considered by the International Bureau as a request for recording a change in the address of the agent under Rule 92bis. In case of disagreement, the applicant should notify the IB accordingly.

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer:
Ingrid Hours

Telephone No.: (41-22) 730.91.11

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
P199500538 WO		
International application No.	International filing date (day/month/year)	Priority date (day month year)
PCT/DK 96/00231	31/05/1996	02/06/1995
International Patent Classification (IPC) or national classification and IPC		
C12N15/10		
Applicant		
Mouritsen & Elsner A/S et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   [X] This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consists of a total of 6 sheets.

3. This report contains indications and corresponding pages relating to the following items:

   - I [X] Basis of the report
   - II [ ] Priority
   - III [ ] Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV [ ] Lack of unity of invention
   - V [X] Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI [ ] Certain documents cited
   - VII [ ] Certain defects in the international application
   - VIII [X] Certain observations on the international application

Date of submission of the demand	Date of completion of this report
23/12/1996	27.10.97
Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	A. Merlos Telephone No.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

I. Basis of the report

1. This report has been drawn up on the basis of (Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.):

[ ] the international application as originally filed.

[x] the description, pages 1-17, as originally filed,
pages _____, filed with the demand,
pages _____, filed with the letter of _____,
pages _____, filed with the letter of _____.

[x] the claims, Nos. _____, as originally filed,
Nos. _____, as amended under Article 19,
Nos. _____, filed with the demand,
Nos. 1-29, filed with the letter of 05.09.97,
Nos. _____, filed with the letter of _____.

[x] the drawings, sheets/fig 1/1, as originally filed,
sheets/fig _____, filed with the demand,
sheets/fig _____, filed with the letter of _____,
sheets/fig _____, filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

[ ] the description, pages _____.
[ ] the claims, Nos. _____.
[ ] the drawings, sheets/fig _____.

3. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims 1,part.-10,part., 13,part.-29, part.	YES
	Claims	NO
Inventive Step (IS)	Claims 1,part.-10,part., 13,part.-29,part.	YES
	Claims	NO
Industrial Applicability (IA)	Claims 1-29	YES
	Claims	NO

2. CITATIONS AND EXPLANATIONS

1). The amended set of claims 1-29 is not in conformity with the requirements of Art. 34(2,b) PCT. In particular, no basis can be found in the original disclosure for a method as claimed in present claim 1 (in particular, for identifying a cellular ligand). The Applicant failed to indicate in his letter of reply where a basis can be found in the original disclosure for the introduced amendments. The Applicant may be of the opinion that for example original claims 1 and 25 support the subject-matter of new claim 1. In this case, it is noted that the amendment is considered as presenting information to the skilled person which is not directly and unambigously derivable from that previously presented in the application, even when account is taken of matter which is implicit to a person skilled in the art. Furthermore, the features as presented in claims 10 and 11 are also not obviously derivable from the original disclosure, neither from figure 1 nor from the corresponding explanations in the description.

2). With respect to the given time limits which were already extended, the Applicant cannot be given another opportunity to file an amended set of claims which meets the requirements of Art. 34(2,b) PCT.
Therefore, examination for novelty and inventive step of the present claims is carried out for a method which refers to the identification of biologically active nucleic acids or peptides (claim 1), further not including the subject-matter of claims 11 and 12.

i) In view of the limited available prior art, it would appear that the subject-matter of claims 1-10, and 13-29 is novel according to Art. 33(2) PCT.

ii) It would further appear that with respect to the prior art cited in the ISR, the idea on which the present invention is based was not obviously and logically derivable. The IPEA is of the opinion that apart from the document cited in the application (page 3), there exist a number of further documents which deal with in vitro selection (SELEX), a technique that allows the simultaneous screening of highly diverse pools of different RNA or (ss, ds) DNA molecules for a particular feature. However, non such documents were cited in the ISR, possibly because they refer to an in vitro selection system. In contrast, the present application is directed to a selection method in an "in vivo" system.

The problem to be solved by the present invention is the identification of biologically active nucleic acids and peptides by use of totally random DNA sequences expressed in an appropriate system. In paricular, according to the present method, a eukaryotic host cell is transformed with a vector containing synthetic random DNA sequences. Upon expression of these sequences, the cells are screened for a resulting biological effect possibly provoked by the synthesized random RNA or the

corresponding random translation product.
None of the documents cited in the ISR discloses means and methods similar to those of the present invention or expresses the need for an in vivo selection system.

Thus, the application does further appear to be based on inventive activity according to Art. 33(3) PCT.

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Most of the claims are not considered to fulfil the requirements of articles 5 and 6 PCT.

Article 6 PCT

The expressions "... in which restrictions upon the randomness may be introduced ...", "purification tag", "... by the principle of ...", "anchor residues", "other leader molecules or recognition sequences" are vague and render the subject-matter claimed open to interpretation.

The protein of claim 22 which is simultaneously expressed from the library vectors lacks any technical definition.
This is also true for the protein of claim 29 which is not sufficiently defined. It is noted that the "random peptide" does not convey any technical or functional characteristics.

The term "coupled to" (claim 1), should be precised (introduced into or fused to).

Art. 5 PCT

The claims refer to subject-matter defined by general features so that the scope of protection claimed appears very broad. The method of claim 1 for example does not define the length of the biologically active nucleic acids or the peptides. It further does not define the vector used nor the eukaryotic cells transformed with said vector.

Having regard to the description, the claims may be considered to enjoy a "formal" support. However, the description lacks sufficient technical information and firm evidence that the selection method actually works in an in vivo system. (It is noted that this is the inventive idea!). One may argue that the invention makes use of conventional methods applied in the art for recombinant expression of peptides or proteins in appropriate host cells, e.g. as described in document WO95/04824 so that the skilled person may carry out the in vivo selection method without undue burden.
On the other hand, even the examples appear to reflect merely a theoretical concept. In particular, they lack any detailed technical data concerning the particular procedure steps and, not to mention, the identification of biologically active nucleic acids or peptides. Moreover, not a single ligand or a drug identified or developped by use of the biologically active nucleic acids or peptides is shown.
In view of the above, the IPEA is therefore of the opinion that the claims are not sufficiently supported by the description (Art. 6 PCT) which in view of the broad field covered is insufficient (Art. 5 PCT).

In view of Art. 5 PCT, it should be clear that the "codon split synthesis" forms part of methods already used in the art at the date of filing (priority date!). In this context, the Applicant's attention is further drawn to the fact that the document submitted with letter of 05.09.97 cannot be taken as support for sufficient disclosure of the "temperature-ligation method" (published after the priority date of the present application).

PATENT CLAIMS

1. A method for identification of biologically active peptides and nucleic acids comprising the following steps: (a) production of a pool of appropriate vectors each containing totally or partly random DNA sequences, (b) efficient transduction of said vectors into a number of identical eukaryotic cells in such a way that a single ribonucleic acid and possibly peptide is expressed or a limited number of different random ribonucleic acids and peptides are expressed by each cell, (c) screening of said transduced cells to see whether some of them have changed a certain phenotypic trait, (d) selection and cloning of said changed cells, (e) isolation and sequencing of the vector DNA in said phenotypically changed cells, and (f) deducing the ribonucleic acid and peptide sequences from the DNA sequence.

2. A method according to claim 1, in which the peptide is a peptide sequence introduced into or fused to a larger protein, preferably a F(ab) fragment or an antibody molecule.

3. A method according to claim 1 or 2, in which the amino acid sequences of the random peptide library are encoded by synthetic DNA sequences/oligonucleotides produced by codon split synthesis, where defined DNA codons are synthesized in a random order.

4. A method according to claim 1 or 2, in which the amino acid sequences of the random peptide library are encoded by synthetic DNA sequences/oligonucleotides produced by conventional random oligonucleotide synthesis.

5. A method according to any one of claims 1-4 in which the random DNA sequences are introduced into the expression vector by the principle of site directed PCR-

mediated mutagenesis hereby ensuring the complexity of the library.

6. A method according to claim 5 in which 3'-5' exonuclease trimming of PCR product 3' ends is used for optimal combining efficiencies of two such PCR products.

7. A method according to any one of claims 1-6, in which temperature-cycling ligation is used for optimal ligation of a DNA fragment into a vector, maintaining a high diversity of the library for transfection into packaging cells.

8. A method according to any one of claims 1-7, in which the random DNA sequences are introduced into the number of eukaryotic cells in such a way that only one DNA sequence is introduced in each cell, one cell expressing one ribonucleic acid and possibly one peptide, thus enabling a particular sequence to by isolated and analyzed.

9. A method according to any one of claims 1-8, in which the random DNA sequences are introduced into the eukaryotic cells by the use of appropriate viral vectors selected from e.g. retrovirus or vaccinia virus.

10. A method according to claim 9, in which the vector used is a retroviral vector.

11. A method according to claim 9 or 10, in which the random DNA sequences are produced as linear PCR products which are directly introduced into the virus packaging cells by non-viral transfection methods.

12. A method according to any one of claims 9-11, in which the viral DNA introduced into the cells is amplified directly by PCR and used for retransfection of new target cells with the purpose of eliminating false posi-

tives and/or enabling the "one cell - one ribonucleic acid or peptide" concept.

13. A method according to any one of claims 9-12, in which the viral titer of retroviral packaging cell lines is increased by transient transfection with a functional tRNA gene corresponding to the PBS in the vector.

14. A method according to any one of claims 9-13, in which a packaging cell line constructed from a vector expressing a single transcript translating the three polyproteins/proteins, gag-pol, a drug resistance gene, and the env gene is used.

15. A method according to any one of claims 9-14, in which a semi-packaging cell line with a corresponding minivirus/vector enabling vector expression after transduction rather than transfection of cells is used.

16. A method according to any one of claims 1-15, in which appropriate restrictions upon the random nature of the expressed peptides are introduced such as e.g. glycosylation sites and anchor residues.

17. A method according to any one of claims 1-16, in which the biologically active peptide or protein also contains a purification tag enabling the direct isolation of the biologically active protein as well as the target protein causing the biological activity.

18. A method according to any one of claims 1-17, in which appropriate signal peptides, other leader molecules or recognition sequences also are encoded by the introduced DNA in such a way that they are fused to the expressed random peptides, or the expressed proteins containing the random peptide sequences, enabling these to be directed towards defined cellular compartments.

19. A method according to any one of claims 1-18, in which the random DNA sequences are introduced into, or fused to a DNA sequence encoding a larger protein expressed simultaneously from the library vectors.

20. A method according to claim 19, in which the larger proteins are selected from secreted proteins, intracellular proteins, and membrane proteins e.g. signal transducing molecules.

21. A method according to claim 19 or 20, in which the larger protein is derived wholly or partly from the heavy and/or light chain of an antibody molecule.

22. A method according to any one of claims 1-21, which is used for identification of T cell epitopes.

23. A method according to any one of claims 1-21, which is used for identifying biologically active peptides which regulate cell surface expression of proteins.

24. Use of a ribonucleic acid or peptide identified by the method according to any one of claims 1-23 as a lead compound for drug development.

25. Use of a ribonucleic acid or peptide identified by the method according to any one of claims 1-23 for isolation of the cellular ligand interacting with said ribonucleic acid or peptide.

26. Use of a larger protein containing a particular amino acid sequence identified by the method according to any one of claims 1-21 for isolation of the cellular ligand interacting with said larger protein.

PCT

LL/MLR

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

Applicant's or agent's file reference *(if desired) (12 characters maximum)* 2950538 WO

Box No. I TITLE OF INVENTION

A method for identification of biologically active peptides and nucleic acids

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

MOURITSEN & ELSNER A/S
Lersø Parkallé 40
DK-2100 Copenhagen Ø
Denmark

☐ This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State *(i.e. country)* of nationality: DK Denmark

State *(i.e. country)* of residence: DK Denmark

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

JENSEN, Martin Roland
Sydskrænten 6
DK-2840 Holte
Denmark

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: DK Denmark

State *(i.e. country)* of residence: DK Denmark

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

Hofman-Bang & Boutard, Lehmann & Ree A/S
Adelgade 15
DK-1304 Copenhagen K
Denmark

Telephone No. 45 33 15 05 85

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Teleprinter No. 19 085 hbb dk

☐ Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Continuation of Box No. III **FURTHER APPLICANTS AND/OR (FURTHER) INVENTORS**

If none of the following sub-boxes is used, this sheet is not to be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

PEDERSEN, Finn Skou
Præstehaven 47
DK-8210 Aarhus V
Denmark

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: DK Denmark

State *(i.e. country)* of residence: DK Denmark

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

MOURITSEN, Søren
Lindevangsvej 24
DK-3460 Birkerød
Denmark

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: DK Denmark

State *(i.e. country)* of residence: DK Denmark

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

HINDERSSON, Peter
Jerichausgade 3
DK-1777 Copenhagen V
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This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: DK Denmark

State *(i.e. country)* of residence: DK Denmark

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

DUCH, Mogens
Elmevej 4
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This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: DK Denmark

State *(i.e. country)* of residence: DK Denmark

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

[X] Further applicants and/or (further) inventors are indicated on another continuation sheet.

Continuation of Box No. III FURTHER APPLICANTS AND/OR (FURTHER) INVENTORS

If none of the following sub-boxes is used, this sheet is not to be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

SØRENSEN, Michael Schandorf
Viborgvej 33, 1. tv.
DK-8000 Aarhus
Denmark

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: DK Denmark

State *(i.e. country)* of residence: DK Denmark

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

DALUM, Iben
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Denmark

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: DK Denmark

State *(i.e. country)* of residence: DK Denmark

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

LUND, Anders Henrik
Rosenkrantzgade 1
DK-8000 Aarhus C
Denmark

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: DK Denmark

State *(i.e. country)* of residence: DK Denmark

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

This person is:
- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality:

State *(i.e. country)* of residence:

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [ ] the United States of America only
- [ ] the States indicated in the Supplemental Box

- [ ] Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No.V **DESIGNATION OF STATES**

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes, at least one must be marked)*:

Regional Patent

- [X] AP **ARIPO Patent:** **KE** Kenya, **LS** Lesotho, **MW** Malawi, **SD** Sudan, **SZ** Swaziland, **UG** Uganda, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- [X] EA **Eurasian Patent:** **AZ** Azerbaijan, **BY** Belarus, **KZ** Kazakstan, **RU** Russian Federation, **TJ** Tajikistan, **TM** Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- [X] EP **European Patent:** **AT** Austria, **BE** Belgium, **CH and LI** Switzerland and Liechtenstein, **DE** Germany, **DK** Denmark, **ES** Spain, **FR** France, **GB** United Kingdom, **GR** Greece, **IE** Ireland, **IT** Italy, **LU** Luxembourg, **MC** Monaco, **NL** Netherlands, **PT** Portugal, **SE** Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- [X] OA **OAPI Patent:** **BF** Burkina Faso, **BJ** Benin, **CF** Central African Republic, **CG** Congo, **CI** Côte d'Ivoire, **CM** Cameroon, **GA** Gabon, **GN** Guinea, **ML** Mali, **MR** Mauritania, **NE** Niger, **SN** Senegal, **TD** Chad, **TG** Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

- [X] AL Albania
- [X] AM Armenia .....
- [X] AT Austria .....
- [X] AU Australia .....
- [X] AZ Azerbaijan
- [X] BB Barbados
- [X] BG Bulgaria .....
- [X] BR Brazil .....
- [X] BY Belarus
- [X] CA Canada
- [X] CH and LI Switzerland and Liechtenstein
- [X] CN China .....
- [X] CZ Czech Republic .....
- [X] DE Germany .....
- [X] DK Denmark .....
- [X] EE Estonia .....
- [X] ES Spain .....
- [X] FI Finland .....
- [X] GB United Kingdom
- [X] GE Georgia .....
- [X] HU Hungary .....
- [X] IS Iceland
- [X] JP Japan .....
- [X] KE Kenya .....
- [X] KG Kyrgyzstan .....
- [X] KP Democratic People's Republic of Korea .....
- [X] KR Republic of Korea .....
- [X] KZ Kazakstan .....
- [X] LK Sri Lanka
- [X] LR Liberia
- [X] LS Lesotho .....
- [X] LT Lithuania
- [X] LU Luxembourg .....
- [X] LV Latvia
- [X] MD Republic of Moldova .....
- [X] MG Madagascar .....
- [X] MK The former Yugoslav Republic of Macedonia .....
- [X] MN Mongolia
- [X] MW Malawi .....
- [X] MX Mexico .....
- [X] NO Norway
- [X] NZ New Zealand .....
- [X] PL Poland .....
- [X] PT Portugal .....
- [X] RO Romania
- [X] RU Russian Federation .....
- [X] SD Sudan
- [X] SE Sweden
- [X] SG Singapore
- [X] SI Slovenia .....
- [X] SK Slovakia .....
- [X] TJ Tajikistan .....
- [X] TM Turkmenistan .....
- [X] TR Turkey .....
- [X] TT Trinidad and Tobago .....
- [X] UA Ukraine .....
- [X] UG Uganda .....
- [X] US United States of America .....
- [X] UZ Uzbekistan .....
- [X] VN Viet Nam .....

Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet:

- [ ] .....
- [ ] .....
- [ ] .....
- [ ] .....

In addition to the designations made above, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except the designation(s) of _____.

The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

Box No. VI PRIORITY CLAIM Further priority claims are indicated in the Supplemental Box ☐

The priority of the following earlier application(s) is hereby claimed:

Country *(in which, or for which, the application was filed)*	Filing Date *(day/month/year)*	Application No.	Office of filing *(only for regional or international application)*
item (1) DK Denmark	(02.06.95) 02 June 1995	0629/95	
item (2)			
item (3)			

Mark the following check-box if the certified copy of the earlier application is to be issued by the Office which for the purposes of the present international application is the receiving Office (a fee may be required).

☐ The receiving Office is hereby requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s) : _____

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(If two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used)*: ISA / SE

Earlier search *Fill in where a search (international, international-type or other) by the International Searching Authority has already been carried out or requested and the Authority is now requested to base the international search, to the extent possible, on the results of that earlier search. Identify such search or request either by reference to the relevant application (or the translation thereof) or by reference to the search request:*

Country (or regional Office): Date *(day/month/year)*: Number:

Box No. VIII CHECK LIST

This international application contains the following number of sheets:

1. request	5	sheets
2. description	17	sheets
3. claims	4	sheets
4. abstract	1	sheets
5. drawings	1	sheets
Total	28	sheets

This international application is accompanied by the item(s) marked below:

1. ☐ separate signed power of attorney
2. ☐ copy of general power of attorney
3. ☐ statement explaining lack of signature
4. ☒ priority document(s) *identified in Box No. VI as item(s):* 1
5. ☒ fee calculation sheet
6. ☐ separate indications concerning deposited microorganisms
7. ☐ nucleotide and/or amino acid sequence listing (diskette)
8. ☐ other *(specify)*:

Figure No. 1 of the drawings (if any) should accompany the abstract when it is published.

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

MOURITSEN & ELSNER A/S
Søren Mouritsen
Man. Director

Martin Roland Jensen
Finn Skou Pedersen
Søren Mouritsen
Peter Hindersson

Mogens Duch
Michael Schandorf Sørensen
Iben Dalum
Anders Henrik Lund

For receiving Office use only

1. Date of actual receipt of the purported international application:	2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:	
4. Date of timely receipt of the required corrections under PCT Article 11(2):	
5. International Searching Authority specified by the applicant: ISA /	6. ☐ Transmittal of search copy delayed until search fee is paid

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

09/854560 ; appl. Ty, 7 449 C.

L7 ANSWER 17 OF 37 EMBASE COPYRIGHT 2001 ELSEVIER SCI. B.V.
ACCESSION NUMBER: 75197371 EMBASE
DOCUMENT NUMBER: 1975197371
TITLE: Ribodeoxyviruses and cancer.
AUTHOR: Temin H.M.
CORPORATE SOURCE: McArdle Lab., Univ. Wisconsin, Madison, Wis. 53706, United States
SOURCE: Journal of the American Medical Association, (1974) 230/7 (1043-1045).
CODEN: JAMAAP
DOCUMENT TYPE: Journal
FILE SEGMENT: 047 Virology
005 General Pathology and Pathological Anatomy
016 Cancer
LANGUAGE: English

AB Ribodeoxyviruses are viruses whose particles (virions) are enveloped and contain RNA and a DNA polymerase. Some of these viruses cause cancer, but most do not, nor do they cause any other disease. Ribodeoxyviruses replicate through a DNA intermediate, the DNA ***provirus*** . The DNA ***provirus*** can be assayed as infectious DNA. Because ribodeoxyvirus information can be present in cells as a part of the cell genome, it needs no viral functions for its maintenance. Therefore, ribodeoxyviruses often persist in cells with little or no viral gene ***expression*** . Most cancers in animals do not contain infectious ribodeoxyviruses, although they may contain ribodeoxyvirus genes and products. These same genes and products are, however, found with about the same frequency in normal cells, so that their presence in human tumors would not indicate an etiologic relation between ribodeoxyviruses and human cancer. It has been suggested that ribodeoxyviruses and the genes for cancer evolve independently from a normal cellular system of DNA to RNA to DNA information transfer.

cur by chromosomal crossing over. This phenomenon enhances genetic reassortment because each of the gametes produced will be genotypically unique.

Diploid organisms reproducing by sexual means can greatly benefit from the fact that one of the two copies of each gene can mutate and serve as the source of new genetic diversity. Beneficial mutations from different individuals of the same species can come together by sexual reproduction and thus be spread quickly among the population. In asexually reproducing procaryotes, on the other hand, independently derived beneficial mutations compete with one another in the two cell populations that spawned them and are not united until a sexual process occurs (if it occurs at all).

Processes similar to sexual reproduction also occur in procaryotes, but by mechanisms that are quite distinct from the process in eucaryotes. First, the process is quite fragmentary, almost never involving whole chromosome complements of the two cells. Second, the DNA is transferred in only one direction, from a donor to a recipient. Third, the mechanisms by which DNA transfer occurs are specialized. Three distinct types of mechanisms for DNA transfer have been recognized: 1) *Conjugation*, in which DNA transfer occurs as a result of cell-to-cell contact. Conjugation is the bacterial process that most closely resembles sex in eucaryotes. 2) *Transduction*, in which DNA transfer is mediated by viruses. 3) *Transformation*, in which free DNA is involved. In transformation, the donor cell generally lyses, releasing DNA into the medium, and some of this free (naked) DNA is taken up by recipient cells. We discuss the details of these various DNA transfer processes in Chapter 7.

Although biologists have struggled for years to understand why sexual reproduction is so successful, the general conclusion is that sexual processes and the reshuffling of genes that it entails, must increase the probability of survival and success of the organism in its environment. In the case of bacteria, it should be pointed out that our knowledge of bacterial sexuality is quite limited. Although laboratory evidence suggests that sexuality is not the norm in bacteria, in nature this may not be the case. Indeed evidence from studies of the bacterial transfer of antibiotic resistance genes located on small genetic elements called plasmids (see Section 5.5) suggest that sexual processes among bacteria in nature may be widespread. Thus, reshuffling of genes by sexual processes in bacteria may actually be an important mechanism for generating genetic diversity in these organisms as well.

3.18 Comparisons of the Procaryotic and Eucaryotic Cell

At this stage it might be useful to draw comparisons between the procaryotic and eucaryotic cell. It should be clear by now that there are profound differences in the structures of these two cell types. One important distinction is that eucaryotes have many types of cellular functions segregated into membrane-containing structures. We discussed mitochondria and chloroplasts earlier and Table 3.4 lists a number of other membranous structures.

Table 3.5 groups these differences into several categories of which the most important are: nuclear structure and function, cytoplasmic structure and organization, and forms of motility.

Table 3.4 Membrane-containing structures in eucaryotes

Structure	Characteristics	Function
Mitochondria	Bacteria-size, complex internal membrane arrays	Energy generation: respiration
Chloroplasts	Green, chlorophyll-containing, many shapes, often quite large	Photosynthesis
Endoplasmic reticulum	Not a distinct organelle, extensive array of internal membranes	Protein synthesis
Golgi bodies	Membrane aggregates of distinct structure	Secretion of enzymes and other macromolecules
Vacuoles	Round, membrane-enclosed bodies of low density	Food digestion: food vacuoles; waste product excretion: contractile vacuoles
Lysosomes	Submicroscopic membrane-enclosed particles	Contain and release digestive enzymes
Peroxisomes	Submicroscopic membrane-enclosed particles	Photorespiration in plants
Glyoxysomes	Submicroscopic membrane-enclosed particles	Enzymes of glyoxylate cycle
Nucleus	Large, generally centrally located	Contains genetic material

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING SUBMISSION OF PRIORITY DOCUMENTS

(PCT Administrative Instructions, Section 411)

To:

HOFMAN-BANG & BOUTARD, LEHMANN & REE A/S
Adelgade 15
DK-1304 Copenhagen K
DANEMARK

Date of mailing (day/month/year) 27 June 1996 (27.06.96)	
Applicant's or agent's file reference 2950538 WO	IMPORTANT NOTIFICATION

International application No.	**International filing date** (day/month/year)	**Priority date** (day month year)
PCT/DK96/00231	31 May 1996 (31.05.96)	02 June 1995 (02.06.95)

Applicant

MOURITSEN & ELSNER A/S et al

The applicant is hereby notified of the date of receipt by the International Bureau of the priority document(s) relating to the following application(s):

Priority application No:	Priority date:	Priority country:	Date of receipt of priority document:
0629/95	02 Jun 1995 (02.06.95)	DK	26 Jun 1996 (26.06.96)

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Mirjam Van Straten

Telephone No.: (41-22) 730.91.11

Form PCT/IB/304 (July 1992)

001134516

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:

HOFMAN-BANG & BOUTARD, LEHMANN & REE A/S
Adelgade 15
DK-1304 Copenhagen K
DANEMARK

Date of mailing (day/month/year)	
05 December 1996 (05.12.96)	
Applicant's or agent's file reference 2950538 WO	**IMPORTANT NOTICE**

International application No.	International filing date (day/month/year)	Priority date (day/month/year)
PCT/DK96/00231	31 May 1996 (31.05.96)	02 June 1995 (02.06.95)

Applicant
MOURITSEN & ELSNER A/S et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AT,AU,BR,CA,CN,CZ,DE,EP,FI,GB,JP,KP,KR,NO,NZ,PL,RO,SK,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AL,AM,AP,AZ,BB,BG,BY,CH,DK,EA,EE,ES,GE,HU,IS,KE,KG,KZ,LK,LR,LS,LT,LU,LV,MD,MG,MK, MN,MW,MX,OA,PT,RU,SD,SE,SG,SI,TJ,TM,TR,TT,UA,UG,UZ,VN

   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   05 December 1996 (05.12.96) under No. WO 96/38553

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No. (41-22) 740.14.35

Authorized officer
J. Zahra
Telephone No. (41-22) 730.91.11

TENT COOPERATION TREA

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
P199500538 WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/DK 96/00231	31/05/1996	02/06/1995
International Patent Classification (IPC) or national classification and IPC		
C12N15/10		
Applicant		
Mouritsen & Elsner A/S et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This **REPORT** consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by **ANNEXES**, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consists of a total of 6 sheets.

3. This report contains indications and corresponding pages relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
23/12/1996	27. 10. 97
Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	A. Merlos Telephone No.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

Intern. application No. PCT/DK96/00231

I. Basis of the report

1. This report has been drawn up on the basis of (Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.):

[ ] the international application as originally filed.

[x] the description, pages 1-17, as originally filed,
pages _____, filed with the demand,
pages _____, filed with the letter of _____,
pages _____, filed with the letter of _____,

[x] the claims, Nos. _____, as originally filed,
Nos. _____, as amended under Article 19,
Nos. _____, filed with the demand,
Nos. 1-29, filed with the letter of 05.09.97,
Nos. _____, filed with the letter of _____,

[x] the drawings, sheets/fig 1/1, as originally filed,
sheets/fig _____, filed with the demand,
sheets/fig _____, filed with the letter of _____,
sheets/fig _____, filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

[ ] the description, pages _____.
[ ] the claims, Nos. _____.
[ ] the drawings, sheets/fig _____.

3. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims 1,part.-10,part., 13,part.-29, part.	YES
	Claims	NO
Inventive Step (IS)	Claims 1,part.-10,part., 13,part.-29,part.	YES
	Claims	NO
Industrial Applicability (IA)	Claims 1-29	YES
	Claims	NO

2. CITATIONS AND EXPLANATIONS

1). The amended set of claims 1-29 is not in conformity with the requirements of Art. 34(2,b) PCT. In particular, no basis can be found in the original disclosure for a method as claimed in present claim 1 (in particular, for identifying a cellular ligand). The Applicant failed to indicate in his letter of reply where a basis can be found in the original disclosure for the introduced amendments. The Applicant may be of the opinion that for example original claims 1 and 25 support the subject-matter of new claim 1. In this case, it is noted that the amendment is considered as presenting information to the skilled person which is not directly and unambigously derivable from that previously presented in the application, even when account is taken of matter which is implicit to a person skilled in the art. Furthermore, the features as presented in claims 10 and 11 are also not obviously derivable from the original disclosure, neither from figure 1 nor from the corresponding explanations in the description.

2). With respect to the given time limits which were already extended, the Applicant cannot be given another opportunity to file an amended set of claims which meets the requirements of Art. 34(2,b) PCT.
Therefore, examination for novelty and inventive step of the present claims is carried out for a method which refers to the identification of biologically active nucleic acids or peptides (claim 1), further not including the subject-matter of claims 11 and 12.

i) In view of the limited available prior art, it would appear that the subject-matter of claims 1-10, and 13-29 is novel according to Art. 33(2) PCT.

ii) It would further appear that with respect to the prior art cited in the ISR, the idea on which the present invention is based was not obviously and logically derivable. The IPEA is of the opinion that apart from the document cited in the application (page 3), there exist a number of further documents which deal with in vitro selection (SELEX), a technique that allows the simultaneous screening of highly diverse pools of different RNA or (ss, ds) DNA molecules for a particular feature. However, non such documents were cited in the ISR, possibly because they refer to an in vitro selection system. In contrast, the present application is directed to a selection method in an "in vivo" system.

The problem to be solved by the present invention is the identification of biologically active nucleic acids and peptides by use of totally random DNA sequences expressed in an appropriate system. In paricular, according to the present method, a eukaryotic host cell is transformed with a vector containing synthetic random DNA sequences. Upon expression of these sequences, the cells are screened for a resulting biological effect possibly provoked by the synthesized random RNA or the

corresponding random translation product.
None of the documents cited in the ISR discloses means and methods similar to those of the present invention or expresses the need for an in vivo selection system.

Thus, the application does further appear to be based on inventive activity according to Art. 33(3) PCT.

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Most of the claims are not considered to fulfil the requirements of articles 5 and 6 PCT.

Article 6 PCT

The expressions "... in which restrictions upon the randomness may be introduced ...", "purification tag", "... by the principle of ...", "anchor residues", "other leader molecules or recognition sequences" are vague and render the subject-matter claimed open to interpretation.

The protein of claim 22 which is simultaneously expressed from the library vectors lacks any technical definition.
This is also true for the protein of claim 29 which is not sufficiently defined. It is noted that the "random peptide" does not convey any technical or functional characteristics.

The term "coupled to" (claim 1), should be precised (introduced into or fused to).

Art. 5 PCT

The claims refer to subject-matter defined by general features so that the scope of protection claimed appears very broad. The method of claim 1 for example does not define the length of the biologically active nucleic acids or the peptides. It further does not define the vector used nor the eukaryotic cells transformed with said vector.

Having regard to the description, the claims may be considered to enjoy a "formal" support. However, the description lacks sufficient technical information and firm evidence that the selection method actually works in an in vivo system. (It is noted that this is the inventive idea!). One may argue that the invention makes use of conventional methods applied in the art for recombinant expression of peptides or proteins in appropriate host cells, e.g. as described in document WO95/04824 so that the skilled person may carry out the in vivo selection method without undue burden.
On the other hand, even the examples appear to reflect merely a theoretical concept. In particular, they lack any detailed technical data concerning the particular procedure steps and, not to mention, the identification of biologically active nucleic acids or peptides. Moreover, not a single ligand or a drug identified or developped by use of the biologically active nucleic acids or peptides is shown.
In view of the above, the IPEA is therefore of the opinion that the claims are not sufficiently supported by the description (Art. 6 PCT) which in view of the broad field covered is insufficient (Art. 5 PCT).

In view of Art. 5 PCT, it should be clear that the "codon split synthesis" forms part of methods already used in the art at the date of filing (priority date!). In this context, the Applicant's attention is further drawn to the fact that the document submitted with letter of 05.09.97 cannot be taken as support for sufficient disclosure of the "temperature-ligation method" (published after the priority date of the present application).

ANNEX

PATENT CLAIMS

1. A method for identification of biologically active nucleic acids or peptides or their cellular ligands, which comprises the steps of (a) production of a pool of appropriate vectors each containing a DNA sequence to be examined, (b) efficient transduction of said vectors into a number of identical eukaryotic cells in such a way that a single ribonucleic acid and possibly peptide is expressed or a limited number of different ribonucleic acids and peptides are expressed by each cell, (c) screening of said transduced cells to see whether some of them have changed a certain phenotypic trait, and (d) selection and cloning of said changed cells, **characterized** in that the pool of appropriate vectors in step (a) contain totally or partly random DNA sequences selected from the group consisting of:

i) synthetic totally random DNA sequences;

ii) synthetic random DNA sequences, in which restrictions upon the randomness may be introduced for the purpose of limiting the number of available sequences and/or for the introduction of post-translational modifications of expressed peptides;

iii) synthetic random DNA sequences like (i) or (ii) coupled to coding sequences of purification tags in order to facilitate the purification and identification of expressed peptides; and

iv) synthetic random DNA sequences like (i), (ii) or (iii) coupled to the coding sequence of a protein;

and that either

(e) the vector DNA in the phenotypically changed cells is isolated and sequenced, and the sequences of the biologically active ribonucleic acids or peptides are deduced from the sequenced vector DNA;

or

(f) the biologically active ribonucleic acids or peptides expressed in the phenotypically changed cells are used directly for isolation of a ligand molecule to said ribonucleic acid or peptide.

2. A method according to claim 1, in which the peptide is a peptide sequence introduced into or fused to a protein, preferably a F(ab) fragment or an antibody molecule.

3. A method according to claim 1 or 2, in which the amino acid sequences of the random peptide library are encoded by synthetic DNA sequences/oligonucleotides produced by codon split synthesis, where defined DNA codons are synthesized in a random order.

4. A method according to claim 1 or 2, in which the amino acid sequences of the random peptide library are encoded by synthetic DNA sequences/oligonucleotides produced by conventional random oligonucleotide synthesis.

5. A method according to any one of claims 1-4 in which the random DNA sequences are introduced into the expression vector by the principle of site directed PCR-mediated mutagenesis hereby ensuring the complexity of the library.

6. A method according to claim 5 in which 3'-5' exonuclease trimming of PCR product 3' ends is used for optimal combining efficiencies of two such PCR products.

7. A method according to any one of claims 1-6, in which temperature-cycling ligation is used for optimal ligation of a DNA fragment into a vector, maintaining a high diversity of the library for transfection into packaging cells.

8. A method according to any one of claims 1-7, in which the random DNA sequences are introduced into the number of eukaryotic cells in such a way that only one DNA sequence is introduced in each cell, one cell expressing one ribonucleic acid and possibly one peptide, thus enabling a particular sequence to by isolated and analyzed.

9. A method according to any one of claims 1-8, in which the random DNA sequences are introduced into the eukaryotic cells by the use of appropriate viral vectors selected from e.g. retrovirus or vaccinia virus.

10. A method according to claim 9, in which the vector used is a retroviral vector.

11. A method according to claim 10, in which the retroviral vector has heterologous ends to facilitate PCR-based generation of the random DNA sequences.

12. A method according to claim 11, in which the heterologous ends contain two different promoters.

13. A method according to any one of claims 10-12, in which the retroviral vector contains a CMV promoter replacing the viral promoter in the 5'-LTR.

14. A method according to any one of claims 9-13, in which the random DNA sequences are produced as linear PCR products which are directly introduced into the virus packaging cells by non-viral transfection methods.

15. A method according to any one of claims 9-14, in which the viral DNA introduced into the cells is amplified directly by PCR and used for retransfection of new target cells with the purpose of eliminating false positives and/or enabling the "one cell - one ribonucleic acid or peptide" concept.

16. A method according to any one of claims 9-15, in which the viral titer of retroviral packaging cell lines is increased by transient transfection with a functional tRNA gene corresponding to the PBS in the vector.

17. A method according to any one of claims 9-16, in which a packaging cell line constructed from a vector expressing a single transcript translating the three polyproteins/proteins, gag-pol, a drug resistance gene, and the env gene is used.

18. A method according to any one of claims 9-17, in which a semi-packaging cell line with a corresponding minivirus/vector enabling vector expression after transduction rather than transfection of cells is used.

19. A method according to any one of claims 1-18, in which appropriate restrictions upon the random nature of the expressed peptides are introduced such as e.g. glycosylation sites and anchor residues.

20. A method according to any one of claims 1-19, in which the biologically active peptide or protein also

contains a purification tag enabling the direct isolation of the biologically active protein as well as the target protein causing the biological activity.

21. A method according to any one of claims 1-20, in which appropriate signal peptides, other leader molecules or recognition sequences also are encoded by the introduced DNA in such a way that they are fused to the expressed random peptides, or the expressed proteins containing the random peptide sequences, enabling these to be directed towards defined cellular compartments.

22. A method according to any one of claims 1-21, in which the random DNA sequences are introduced into, or fused to a DNA sequence encoding a protein expressed simultaneously from the library vectors.

23. A method according to claim 22, in which the protein is selected from the group consisting of secreted proteins, intracellular proteins, and membrane proteins e.g. signal transducing molecules.

24. A method according to claim 22 or 23, in which the protein is derived wholly or partly from the heavy and/or light chain of an antibody molecule.

25. A method according to any one of claims 1-24, which is used for identification of T cell epitopes.

26. A method according to any one of claims 1-24, which is used for identifying biologically active peptides which regulate cell surface expression of proteins.

27. Use of a ribonucleic acid or peptide identified by the method according to any one of claims 1-26 as a lead compound for drug development.

28. Use of a ribonucleic acid or peptide identified by the method according to any one of claims 1-26 for isolation of a cellular ligand interacting with said ribonucleic acid or peptide.

29. Use of a protein containing a particular amino acid sequence identified by the method according to any one of claims 1-24 for isolation of a cellular ligand interacting with said particular amino acid sequence contained in said protein.

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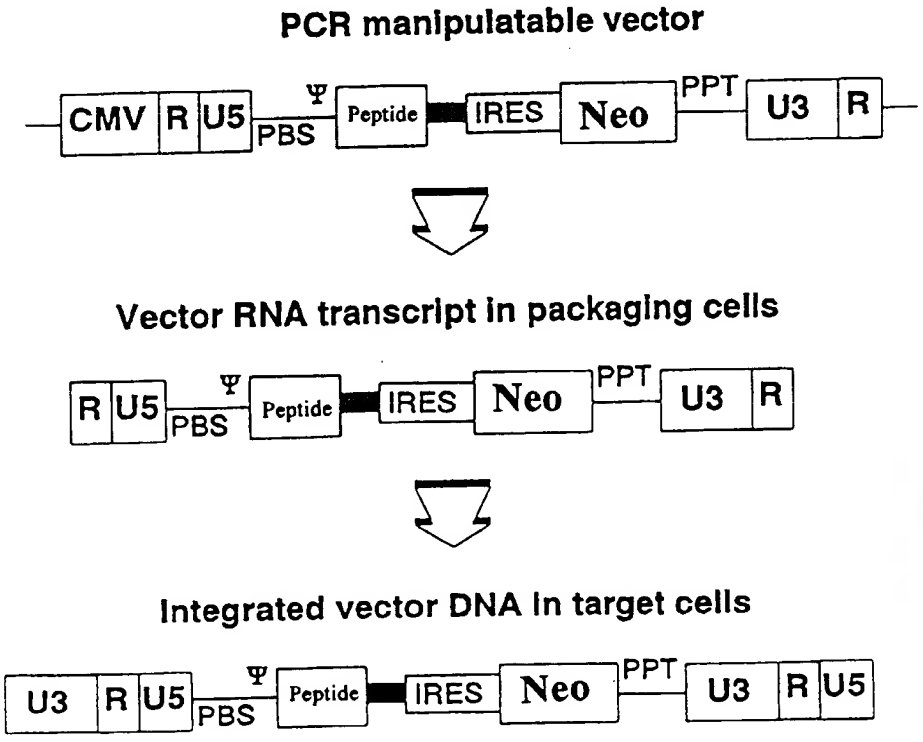
(54) Title: A METHOD FOR IDENTIFICATION OF BIOLOGICALLY ACTIVE PEPTIDES AND NUCLEIC ACIDS

(57) Abstract

Biologically active peptides and nucleic acids are identified by a method comprising the following steps: (a) production of a pool of appropriate vectors each containing totally or partly random DNA sequences, (b) efficient transduction of said vectors into a number of identical eukaryotic cells in such a way that a single ribonucleic acid and possibly peptide is expressed or a limited number of different random ribonucleic acids and peptides are expressed by each cell, (c) screening of said transduced cells to see whether some of them have changed a certain phenotypic trait, (d) selection and cloning of said changed cells, (e) isolation and sequencing of the vector DNA in said phenotypically changed cells, and (f) deducing the ribonucleic acid and peptide sequences from the DNA sequence. The peptide sequences may be introduced into or fused to a larger protein preferably an antibody molecule or a fragment thereof. This may be obtained by introducing the random DNA sequences into or fusing them to a DNA sequence encoding such larger protein.



A METHOD FOR IDENTIFICATION OF BIOLOGICALLY ACTIVE PEPTIDES AND NUCLEIC ACIDS

This invention concerns a novel method for identification of new peptides and post-translationally modified peptides as well as nucleic acids with biological activity.

BACKGROUND OF THE INVENTION

During the last five years the technology for expressing, testing and identifying millions of different random peptide sequences has evolved dramatically. Such peptide libraries can be used for identification of new biologically active peptides, and therefore the technology has added an exciting and very promising new epoch to the field of drug development.

The known peptide library techniques can at present be divided into two fundamentally different groups: The random synthetic peptide libraries, in which the random peptides are produced chemically, and the random biosynthetic peptide libraries, in which the random peptides are encoded by partly or totally random DNA sequences and subsequently synthesized by ribosomes.

The synthetic peptide libraries.

Synthetic peptide libraries containing millions of peptides can be produced by combinatorial peptide chemistry and may either be synthesized in soluble form (R.A. Houghten et al. Nature, 354, 84-86, 1991) or remain immobilized on the peptide resin beads (A. Furka et al., Int. J. Peptide Protein Res. 37, 487-493, 1991; K. S. Lam et al., Nature, 354, 82-84, 1991). Using either of these approaches different receptor ligands have been isolated.

The advantage of the soluble peptide libraries compared to solid phase immobilized peptide libraries is that soluble peptides may bind more sterically unhindered to the receptors in question. In the synthetic peptide library technique proposed by Furka et al. 1991 and Lam et al. 1991, respectively, the most important improvement, on the other hand, was the approach of having only one type of peptide sequence on each bead ("One bead - one peptide"). This enables direct selection and eventually sequencing of the putative active peptide ligand on a single bead using e.g. Edman degradation. Using this technology active peptides including peptides consisting of D-amino acids or other unnatural amino acids can be identified (B. Gissel et al. J. Peptide Science. In Press, 1995).

The biosynthetic peptide libraries.

Bacteriophage expression vectors have been constructed that can display peptides on the phage surface (S.E. Cwirla et al, Proc. Natl. Acad. Sci. USA, 87, 6378-6382, 1990). Each peptide is encoded by a randomly mutated region of the phage genome, so sequencing of the relevant DNA region from the bacteriophage found to bind a receptor will reveal the amino acid sequence of the peptide ligand. A phage containing a peptide ligand is detected by repeated panning procedures which enrich the phage population for a strong receptor binding phage (J.K. Scott, TIBS, 17, 241-245, 1992).

Bacteria have also been used for expression of similar peptide libraries. The peptides can be fused to an exported protein, such as an antibody, which can be immobilized on a solid support. By screening the solid support with an appropriate soluble receptor the bacterial clone producing the putative peptide ligand can be identified (M.G. Cull et al, Proc. Natl. Acad. Sci. USA, 89, 1865-1869, 1992).

Another described method for preparing large pools of different possibly active compounds is by the use of libraries consisting of randomized ribonucleotides or deoxyribonucleotides, the so-called aptamer libraries. The aptamers are generated in E. coli from a plasmid vector containing randomized DNA. From these libraries structures with biological activity have been identified (L.C. Bock et al, Nature, 355, 564-566, 1992).

PURPOSE OF THE INVENTION

In order to use the prior art methods for identifying biologically active peptides and nucleic acids or their respective cellular target proteins, it is necessary to possess a detailed knowledge about the molecular mechanisms involved in a certain biological process. If these mechanisms are known, it may subsequently be possible to develop antagonists or agonists of targets (receptors, enzymes, etc.) involved using said methods. The problem to be solved by the present invention is i.a. to overcome the need for said detailed knowledge.

SUMMARY OF THE INVENTION

According to the present invention the peptide sequences, or the ribonucleic acids are identified from biosynthetically expressed eukaryotic libraries containing millions of partly or totally random peptides and ribonucleic acids. In connection with this invention the term "peptide" shall be understood to comprise also a peptide sequence introduced into or fused to a larger protein (e.g. an antibody). The peptides and ribonucleic acids are synthesized by the cells from random DNA sequences which have been effectively transduced into the cells. Some of the peptides or ribonucleic acids in the library will affect important biological functions in the cells which express them. Cells which change phenotype due to the presence of such substances can be isolated, and their chemical

structure can subsequently be clarified by sequencing of the DNA which encodes them. Such peptides could possibly be used therapeutically or as lead compounds for future drug development or they can be used for identification of new target proteins which are causing the change in the biological function of the cell. Such target proteins can eventually be used in the development of drugs by e.g. conventional medicinal chemistry or synthetic peptide libraries.

Accordingly, the method of the invention comprises the following steps: (a) production of a pool of appropriate vectors each containing totally or partly random DNA sequences, (b) efficient transduction of said vectors into a number of identical, e.g. mammalian, cells in such a way that a single ribonucleic acid and possibly peptide is expressed or a limited number of different random ribonucleic acids and peptides are expressed by each cell, (c) screening of said transduced cells to see whether some of them have changed a certain phenotypic trait, (d) selection and cloning of said changed cells, (e) isolation and sequencing of the vector DNA in said phenotypically changed cells, and (f) deducing the RNA and peptide sequences from the DNA sequence.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is a schematic drawing of a standard retroviral peptide expression vector. The plasmid form of the vector (top) carries a cytomegalovirus (CMV) promoter directing expression of a retroviral RNA (middle) with a backbone (R, U5, PBS, @, PPT, U3, and R) from Akv murine leukaemia virus and a peptide translation cassette followed by an internal ribosomal entry site (IRES) from EMC virus directing translation of a Neomycin resistance gene (Neo). The vector provirus in the target cell (below) contains a regenerated retroviral long terminal repeat - LTR (U3, R, and U5). The CMV promoter sequence provides a unique tag

for efficient initial PCR mutagenesis and amplification. The size of the vector without an inserted peptide expression cassette is 4.0 kb.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

A built-in requirement of all the presently known peptide library techniques is the necessity of a detailed knowledge about the mechanisms by which a given receptor or enzyme regulates a certain phenotypic trait of the cell. This receptor or enzyme furthermore has to be available in a relatively pure form before a ligand can be selected in either of the two types of peptide libraries. When potential peptide ligands eventually have been identified, functional assays have to be performed to determine whether the ligands exert antagonistic or agonistic effects on the desired cellular phenotypic trait.

The method according to the present invention overcomes this major problem. By the present method it is thus not necessary to know the chain of mechanisms, receptors, signalling pathways, enzymes etc. which generate the phenomenon inside or on the surface of the cell since it is the resulting biological effect or phenotypic trait which is screened for. This is achieved by the following steps: (a) production of a pool of appropriate vectors each containing totally or partly random DNA sequences, (b) efficient transduction of said vectors into a number of identical eukaryotic, e.g. mammalian, cells in such a way that only a single ribonucleic acid and peptide species is expressed ("one cell - one ribonucleic acid or peptide") or optionally a limited number of different random ribonucleic acids and peptides are expressed by each cell, (c) screening of said transduced cells to see whether some of these have changed a certain phenotypic trait, (d) selection and cloning of said changed cells, (e) isolation and sequencing of the vector DNA in said phenotypically changed cells, and (f) deducing the RNA

and peptide sequences from the DNA sequence. Either the RNA or the peptide encoded by the isolated DNA sequences may be the cause of said phenotypic changes of the cell and may therefore possess biological activity.

The introduced peptides are expressed e.g. in the cytoplasm of biologically interesting cells, which before that were totally identical, using e.g. the retroviral vector systems described in Example 1. They are expressed from a pool of vectors containing random DNA sequences which has been constructed e.g. as described in Example 1. Using an appropriate ratio between infective retrovirus and non-infected cells only a single DNA copy derived from the pool of retrovirus vectors is introduced into each cell. The major advantage by this "one cell - one ribonucleic acid or peptide" concept is that cells which have changed phenotypically upon the introduction of peptides can be isolated by cloning and selection methods, and that the active peptide causing the phenotypic change can subsequently be identified. This is accomplished by isolating the DNA fragment encoding the peptide, e.g. by Polymerase Chain Reaction (PCR) technology, and subsequently identifying the DNA sequence.

During the initial screening procedure a larger number of retrovirus vectors can be introduced into each cell which enables the individual cell to express a number of different ribonucleic acids or peptides. When a phenotypically changed cell clone subsequently has been isolated all retroviral DNA in that particular clone can be isolated by PCR, and the PCR product can be used for retransfection of the packaging cells ordinarily used for virus production. The retroviral vectors isolated from these packaging cells can subsequently be used for transduction of new biologically interesting cells using the "one cell - one ribonucleic acid or peptide" concept. Finally, after a second cloning procedure the active substance can be identified as described above. Further, the

biologically active ribonucleic acid, peptide or protein isolated by this method can be utilized either as a bad compound for drug development or as an affinity ligand with the purpose of isolating and identifying the protein target responsible for the biological activity. Such target proteins are very useful tools in drug development.

The use of small vectors (below 3 kb of DNA) has the major advantage of allowing simple PCR-mutagenesis and amplification without ligation and cloning steps. Another important advantage of using small vectors is that the vectors in a pool of target cells can be amplified directly by PCR and retransfected into packaging cells, hence allowing multiple rounds of selection to remove time-consuming analysis of false positives or contaminating cells. Direct sequence analysis of the derived random plasmid clones is used to assure the randomness of the expression library. The standard vector capable of expressing the random peptide library is shown schematically in Fig. 1.

The cells which are found to have changed phenotypically upon the introduction of the random DNA sequences could alternatively have changed as a consequence of interactions with the ribonucleic acid molecule transcribed from the introduced DNA, in analogy to the described libraries consisting of randomized ribonucleotides or deoxyribonucleotides, the so-called aptamer libraries. Such ribonucleic acid molecules would therefore also possess biological activity. Furthermore, the observed effect could also be due to biological activity of carbohydrate moieties or other post-translational modifications on the expressed peptides in the cell. Using an appropriate purification-tag on the peptides in the library, it would be possible in that case to purify these and analyze the exact chemical structure of the post-translational modifications in question.

Since the efficiency of the non-viral methods commonly used for stable gene transfer into mammalian cells is very low, it would not be possible to establish a peptide expression library in mammalian cells by such methods. In addition non-viral methods generally lead to multiple integrations of DNA in the cell genome in disagreement with the "one cell one ribonucleic acid or peptide" concept. In order to achieve the necessary high efficiency single gene copy transfer a viral vector must be used. Very recently cDNA expression libraries which were constructed using retroviral vectors have been described. From such libraries cytokines and cellular growth factors have been isolated (A.J.M. Murphy et al., Proc. Natl. Acad. Sci. USA, 84, 8277-8281, 1987., B.Y. Wong et al., J. Virol., 68, 5523-31, 1994., J.R. Rayner et al., Mol. Cell. Biol., 14, 880-887, 1994). Expression of well defined peptides in transfected eukaryotic cells has also previously been established, although not using retroviral vectors (M.S. Malnati et al., Nature, 357, 702-704, 1992., E.O. Long et al., J. Immunol., 153, 1487-1494, 1994). A library of random peptides has never been expressed in mammalian cells with the purpose of identifying biologically active peptides or ribonucleic acids.

Immunology is an important biological field where the method according to the invention can be used. T cells only recognize fragments of protein antigens, and only if these are bound to MHC molecules. Two types of MHC molecules - the class I and II molecules - present antigen fragments to T cells. The peptides presented by MHC class I molecules, which are on the surface of essentially all nucleated cells, are 8-9 amino acids long and generally derived from proteins in the cytosol of the cell. These can be self-proteins, viral proteins, peptides introduced into the cytosol by transfection or tumor antigens. It is of considerable interest to be able to identify such peptides or T cell epitopes e.g. with regard to vaccine development or in immunotherapy of cancer. Identification

of such fragments is, however, a very difficult, demanding and some times impossible task requiring large amounts of affinity purified MHC molecules derived from the tumor cell in question and iterative combinations of advanced mass spectrometry, HPLC and functional T cell assays. Furthermore, most peptide antigens cannot be identified due to the presence of very low amounts of the individual peptides on the MHC molecules. In Example 2 it is demonstrated that the method according to the present invention can be used for identification of said T cell epitopes.

Cell lines expressing biologically important surface molecules can also be transduced with a random peptide library according to the invention. An example of such molecules could be the B7 co-stimulatory molecule, which is known to be important for activation of T cells, or the selectin family of proteins which are known to be involved in the homing of inflammatory cells to inflamed tissue. Cells which change phenotype (e.g. either up- or down-regulate B7) can be selected and cloned as described in Example 3. After isolation of the transduced DNA by PCR new cells can be transduced with the isolated DNA to confirm the observation. Subsequently, the peptide sequence can be deduced from the DNA sequence.

It has been described by others that specific monoclonal antibodies or F(ab) fragments can be expressed in the cytoplasm of a cell and exert a biological activity there (T.M. Werge et al., Febs Letters, 274, 193-198, 1990).

According to the present invention the wholly or partly random peptide sequence can also be introduced into the variable region of an antibody F(ab) fragment. Therefore, a library of F(ab) fragments containing random peptide sequences can be expressed in a cell clone in a way that each all express a single antibody specificity. Subsequently biologically changed cells are isolated and

cloned, and the identified intracellular F(ab) fragment can be used for purification of the target protein involved in the biological proteins. Such target protein can subsequently be used for development of drugs capable of modifying said target proteins.

The invention is illustrated by the following examples:

EXAMPLE 1

Construction of an intracellular eukaryotic peptide library

A retrovirus vector capable of expressing random peptide sequences is constructed. If the random DNA sequences used in the vector were produced using conventional random oligonucleotide synthesis a large number of stop codons inevitably would be introduced. Furthermore, due to the degeneracy of the genetic code an uneven distribution of the encoded amino acids would be the result. We avoid this by producing the random DNA sequences by random codon synthesis: An appropriate amount of resin used for solid phase oligonucleotide synthesis (optionally already containing a DNA sequence corresponding to an appropriate vector cloning site) is divided into 20 different portions. On portion no. 1 a conventional solid phase chemical synthesis of three bases corresponding to a codon encoding the amino acid, alanine, is performed. On portion no. 2 a codon encoding cysteine is synthesized and so forth. When each of the 20 portions have been coupled with codons corresponding to each of the natural amino acids, all portions are mixed and divided again into 20 equally sized resin portions. The codon synthesis is then repeated again, and the whole procedure is repeated until the desired randomized DNA sequence has been synthesized - e.g. corresponding to 6-10 random amino acids. This can also be achieved by using blocked and protected trinucleotide phosphoramidites encoding the 20

natural amino acids in a total random oligonucleotide synthesis (J. Sondek et al., Proc. Natl. Acad. Sci. USA, 89, 3581-5, 1992). Finally, other appropriate vector cloning sites can be synthesized on all the oligonucleotides before they eventually are cleaved from the resin.

The pool of random synthetic oligonucleotides can be used to generate a pool of vectors with random sequences in appropriate positions either by restriction cleavage and ligation or, preferentially, to avoid inefficient ligation steps, by PCR-mutagenesis. The procedures follow the principles of site-directed PCR-mediated mutagenesis (S. Perrin et al., Nucl. Acids Res. 18, 7433-38, 1990), but the methodology has been adapted to deliver a complex mixture of products. Briefly, the random oligonucleotides carrying vector sequences flanking the random sequence are used as primers in a PCR reaction together with a unique terminal vector primer. In order to retain complexity large quantities of template as well as of vector and randomized primers are used, and product diversity is further ensured by pooling of multiple independent PCR-reactions. Subsequently, an overlapping PCR fragment containing the remaining vector segment is produced by standard PCR. Finally, this overlapping segment is joined with the PCR fragments containing the random DNA using another unique set of terminal primers.

DNA fragments produced by Taq DNA polymerase enzyme may contain additional nucleotides at the 3'DNA strand. These extra nucleotides will be deleterious for combining two PCR products with overlapping termini into one fragment. By addition of Klenow DNA polymerase enzyme these nucleotides can be removed by the 3' go 5' exonuclease activity of said enzyme, increasing the combining efficiency.

Alternatively the PCR product can be trimmed at the termini by digestion with restriction enzymes whose recognition sequences have been incorporated into the oligonu-

cleotides used as primers for the PCR reaction. By utilization of the temperature cycle method developed by (Lund et al., Nucleic Acids Res., 24, 800-801, 1996) the efficiency of the ligation reaction can be increased and the ligation product used for direct transfection of the packaging cells.

To maintain diversity, the PCR-generated linear vector DNA is used directly for transfection of packaging cells, and virions containing the pool of different vectors are harvested under transient conditions. Small bicistronic single transcript vectors containing a random peptide translation cassette followed by an internal ribosomal entri site (IRES) from EMC virus directing translation of a Neomycin (Neo) resistance gene. The Neo gene functions as a selection marker to allow titre determination and elimination of non-transduced cells, if necessary. Other available relevant vectors employ other selectable genes such as phleomycin and hygromycin B resistance and have the peptide translation cassette after the IRES element. Alternatively, even smaller vectors, carrying only the peptide expression cassette and lacking a selection marker can also be used.

The use of small vectors (below 4 kb of DNA) has the major advantage of allowing simple PCR-mutagenesis and amplification without ligation and cloning steps. Another important advantage of using small vectors is that the vectors in a pool of target cells can be amplified directly by PCR and retransfected into packaging cells, hence allowing multiple rounds of selection to remove time-consuming analysis of false positives or contaminating cells. Direct sequence analysis of the derived random plasmid clones is used to assure the randomness of the expression library. The standard vector capable of expressing the random peptide library is shown schematically in Fig. 1.

To maintain diversity of the vector pool a high fraction of the vector RNA transcripts must be encapsidated into retroviral particles. By transfection of the packaging cells with a DNA construct expressing a tRNA matching the corresponding PBS in the retroviral vector we can increase the production of functional vector containing virus particles 10 fold under transient conditions..

A new packaging cell line will be generated after a single transfection of a construct encoding all retroviral proteins. To diminish the risk of generation of replication competent virus and to obtain maximal expression the vector will have the following simplified outline: promoter-gag-pol transcript-IRES-phleomycin resistance gene-IRES-env-polyadenylation signal. One advantange of said vector is that the phleomycin resistance gene enables selection for high expression and that the sheer size of the vector transcript restricts the encapsidation into retroviral particles thereby diminishing the risk of generation of replication competent virus. The size limit for encapsidation of RNA transcripts is about 10 kb.

In addition to traditional packaging cell line a semi-packaging cell line with a corresponding minivirus-vector will be used. The semi-packaging cell line consists of vectors encoding two mutated gag-pol transcripts complementing each other. The use of two different gag-pol transcripts reduces the risk of generating wild type virus. Each cell in the semi-packaging cell line now contains all retroviral proteins needed for production of retroviral particles except the envelope proteins these proteins are supplied by the minivirus-vector. This vector is a bicistronic vector with following outline LTR-PBS-packaging signal-random peptide-IREs-env-polypurine tract-LTR. This vector will be able to transduce the semi-packaging cells as these do not produce envelope proteins prior to infection with the minivirus-vector.

Thus, infection of the semi-packaging cell will not be restricted by receptor interference.

Restrictions upon the "randomness" of the peptide sequence can be introduced, for the purpose of limiting the number of available sequences in the mammalian cellular library and for introduction of e.g. N-glycosylation sites, or other post-translational modifications of all expressed peptides if so desired. Purification tags - e.g. poly-His or others - can also be included in the expressed peptides for facilitating the purification and identification of the peptide itself. This is necessary for the identification of post-translational modifications, which were not obvious from the peptide sequence.

A population of eukaryotic cells is infected with the retrovirus carrying genetic constructs containing random DNA sequences which encode a library of millions of random peptides. Initially an excess number of virus compared to eukaryotic cells can be used. This leads to expression of a number of different peptides within each eukaryotic cell. These cells can subsequently be screened as described below, and the DNA can be isolated e.g. by PCR and used for reinfection of other cells. If an appropriate ratio between the number of retrovirus containing the random DNA sequences and cells is chosen, each cell will be transduced with a different random DNA sequence ("one cell - one ribonucleic acid or peptide"). This eventually enables the identification of an active peptide.

The peptide may optionally be targeted to different compartments in the cell by incorporating appropriate signal sequences in the translated sequences.

EXAMPLE 2

Identification of T cell epitopes by the use of mammalian intracellular expression libraries

The interleukin 2 (Il-2) dependent cell line, CTLL-2, is transfected with the murine Major Histocompatibility (MHC) class I molecule, K^b. Subsequently this cell line is infected with a retrovirus peptide library which was described in Example 1. This CTLL-2 peptide library expresses a wide range of random peptides, and if appropriate K^b associated anchor residues known to be important for peptide binding to K^b are introduced in the retroviral peptide sequence, a large library of peptides bound to K^b are presented on the surface of the CTLL-2 cells.

K^b restricted T cell hybridomas are generated against an appropriate virus antigen using conventional cellular immunological technology (Current Protocols in Immunology, Eds. Coligan et al., NIH). Such hybridomas secrete Il-2 upon recognition of antigen. A T cell hybridoma, which recognizes an unknown K^b bound virus T cell epitope, is subsequently incubated with samples of the K^b CTLL-2 library. If the hybridoma recognizes a peptide, the CTLL-2 cell presenting the peptide will be stimulated to proliferate by the Il-2 secreted by the hybridoma. In that way the CTLL-2 cell expressing the unknown virus T cell epitope can be selected and cloned. From this clone the DNA sequence encoding the peptide epitope in question can be isolated using PCR technology followed by conventional DNA sequencing. This eventually leads to identification of the unknown virus T cell epitope.

EXAMPLE 3

Identification of biologically active peptides or ribonucleic acids which regulate cell surface expression of proteins

Cells expressing the immunoregulatory membrane molecule, B7, are infected with the random peptide libraries constructed as described in Example 1. In this example a random eight-mer peptide library is introduced.

Using specific monoclonal antibodies the expression of B7 is analyzed by conventional methods. Cells which up- or down-regulate B7 can be selected either positively, e.g. using Fluorescence Activated Cell Sorting, or negatively, e.g. using appropriate antibodies in combination with lysis by complement.

Cells which show changes in expression of B7 are cloned by conventional means, and the DNA introduced by retroviral vectors is isolated using PCR and a set of retrovirus specific primers. The peptide sequence or possibly the RNA corresponding to said DNA may be able to modify the expression of B7 and hence the activation of T cells. This can subsequently be tested in conventional T cell assays.

EXAMPLE 4

Identification of a F(ab) fragment capable of modifying the immunoregulatory molecule B7.

A retroviral library encoding the variable heavy chain (V_H) as well as the variable light (V_L) gene fragments of the immunoglobulin molecule is produced. The gene region of both gene fragments corresponding to the antigen binding site of the resulting F(ab) fragments contains furthermore partly random gene sequences as described in example 1. This will lead to a large number of diverse peptide sequences in the antigen binding site of the F(ab) fragment. The retroviral vector library therefore encodes a large number of different F(ab) fragments with different antigen binding specification.

This library is subsequently transduced into a cell clone in such a way that each cell expresses a single F(ab) fragment species in e.g. the cytoplasm. Phenotypically changed cells are subsequently cloned and the sequence of the peptide in the intracellular (Fab) fragment is identified as described in example 1. This antibody can subsequently be produced in large scale by conventional means and be used for affinity purification of the cellular target protein responsible for the biological change of the cell phenotype. This can e.g. be done from lysates produced from the original non-modified cell clone.

Alternatively the retroviral F(ab) library can be constructed using e.g. a poly-His tag or other appropriate tags. In that way the antibody and the corresponding target can be isolated directly from the phenotypically changed cell by affinity chromatography. The isolated target can subsequently be identified by e.g. N-terminal amino acid sequencing in combination with conventional cloning methodology. Such target proteins are very important drug targets for further drug discovery.

PATENT CLAIMS

1. A method for identification of biologically active peptides and nucleic acids comprising the following steps: (a) production of a pool of appropriate vectors each containing totally or partly random DNA sequences, (b) efficient transduction of said vectors into a number of identical eukaryotic cells in such a way that a single ribonucleic acid and possibly peptide is expressed or a limited number of different random ribonucleic acids and peptides are expressed by each cell, (c) screening of said transduced cells to see whether some of them have changed a certain phenotypic trait, (d) selection and cloning of said changed cells, (e) isolation and sequencing of the vector DNA in said phenotypically changed cells, and (f) deducing the ribonucleic acid and peptide sequences from the DNA sequence.

2. A method according to claim 1, in which the peptide is a peptide sequence introduced into or fused to a larger protein, preferably a F(ab) fragment or an antibody molecule.

3. A method according to claim 1 or 2, in which the amino acid sequences of the random peptide library are encoded by synthetic DNA sequences/oligonucleotides produced by codon split synthesis, where defined DNA codons are synthesized in a random order.

4. A method according to claim 1 or 2, in which the amino acid sequences of the random peptide library are encoded by synthetic DNA sequences/oligonucleotides produced by conventional random oligonucleotide synthesis.

5. A method according to any one of claims 1-4 in which the random DNA sequences are introduced into the expression vector by the principle of site directed PCR-

mediated mutagenesis hereby ensuring the complexity of the library.

6. A method according to claim 5 in which 3'-5' exonuclease trimming of PCR product 3' ends is used for optimal combining efficiencies of two such PCR products.

7. A method according to any one of claims 1-6, in which temperature-cycling ligation is used for optimal ligation of a DNA fragment into a vector, maintaining a high diversity of the library for transfection into packaging cells.

8. A method according to any one of claims 1-7, in which the random DNA sequences are introduced into the number of eukaryotic cells in such a way that only one DNA sequence is introduced in each cell, one cell expressing one ribonucleic acid and possibly one peptide, thus enabling a particular sequence to by isolated and analyzed.

9. A method according to any one of claims 1-8, in which the random DNA sequences are introduced into the eukaryotic cells by the use of appropriate viral vectors selected from e.g. retrovirus or vaccinia virus.

10. A method according to claim 9, in which the vector used is a retroviral vector.

11. A method according to claim 9 or 10, in which the random DNA sequences are produced as linear PCR products which are directly introduced into the virus packaging cells by non-viral transfection methods.

12. A method according to any one of claims 9-11, in which the viral DNA introduced into the cells is amplified directly by PCR and used for retransfection of new target cells with the purpose of eliminating false posi-

tives and/or enabling the "one cell - one ribonucleic acid or peptide" concept.

13. A method according to any one of claims 9-12, in which the viral titer of retroviral packaging cell lines is increased by transient transfection with a functional tRNA gene corresponding to the PBS in the vector.

14. A method according to any one of claims 9-13, in which a packaging cell line constructed from a vector expressing a single transcript translating the three polyproteins/proteins, gag-pol, a drug resistance gene, and the env gene is used.

15. A method according to any one of claims 9-14, in which a semi-packaging cell line with a corresponding minivirus/vector enabling vector expression after transduction rather than transfection of cells is used.

16. A method according to any one of claims 1-15, in which appropriate restrictions upon the random nature of the expressed peptides are introduced such as e.g. glycosylation sites and anchor residues.

17. A method according to any one of claims 1-16, in which the biologically active peptide or protein also contains a purification tag enabling the direct isolation of the biologically active protein as well as the target protein causing the biological activity.

18. A method according to any one of claims 1-17, in which appropriate signal peptides, other leader molecules or recognition sequences also are encoded by the introduced DNA in such a way that they are fused to the expressed random peptides, or the expressed proteins containing the random peptide sequences, enabling these to be directed towards defined cellular compartments.

19. A method according to any one of claims 1-18, in which the random DNA sequences are introduced into, or fused to a DNA sequence encoding a larger protein expressed simultaneously from the library vectors.

20. A method according to claim 19, in which the larger proteins are selected from secreted proteins, intracellular proteins, and membrane proteins e.g. signal transducing molecules.

21. A method according to claim 19 or 20, in which the larger protein is derived wholly or partly from the heavy and/or light chain of an antibody molecule.

22. A method according to any one of claims 1-21, which is used for identification of T cell epitopes.

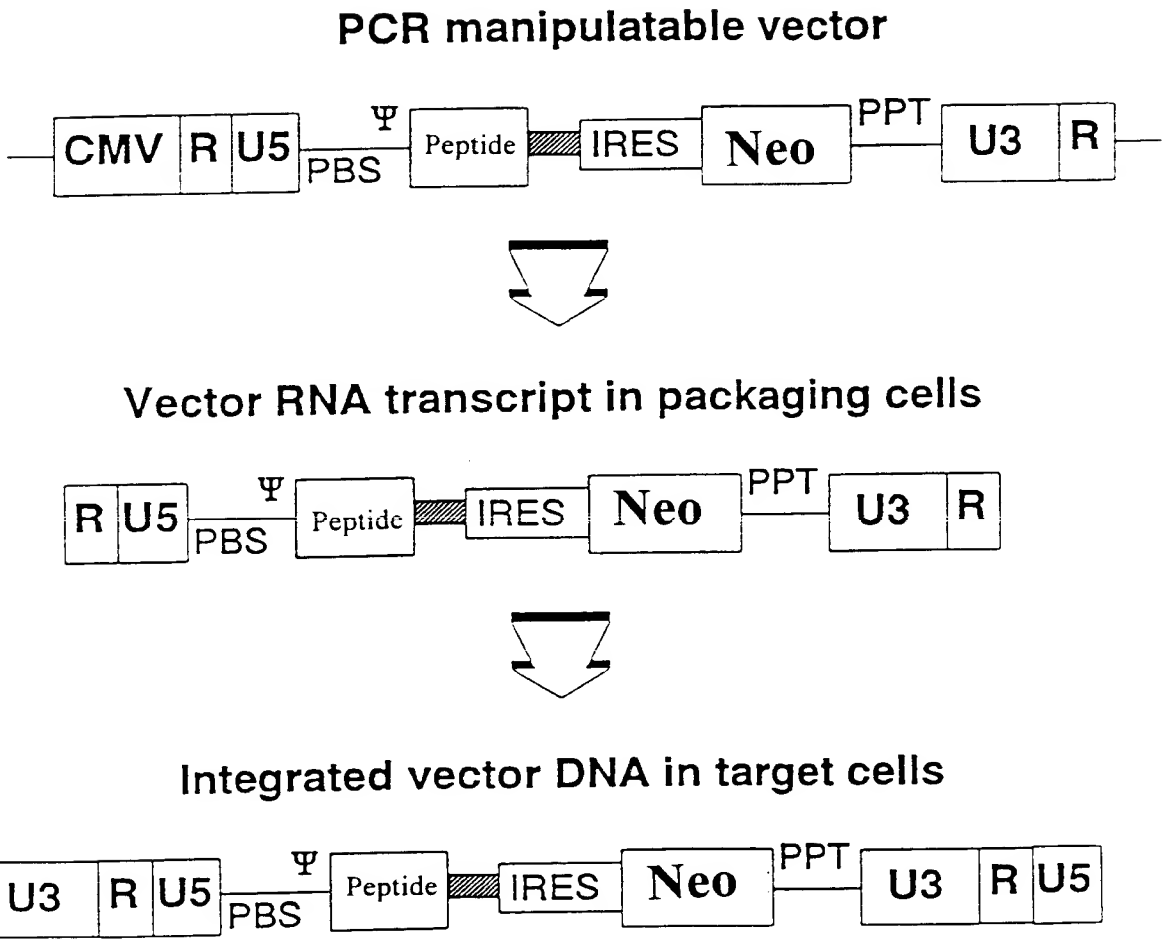
23. A method according to any one of claims 1-21, which is used for identifying biologically active peptides which regulate cell surface expression of proteins.

24. Use of a ribonucleic acid or peptide identified by the method according to any one of claims 1-23 as a lead compound for drug development.

25. Use of a ribonucleic acid or peptide identified by the method according to any one of claims 1-23 for isolation of the cellular ligand interacting with said ribonucleic acid or peptide.

26. Use of a larger protein containing a particular amino acid sequence identified by the method according to any one of claims 1-21 for isolation of the cellular ligand interacting with said larger protein.

Figure 1


PCR manipulatable vector
CMV
R
U5
Ψ
PBS
Peptide
IRES
Neo
PPT
U3
R
Vector RNA transcript in packaging cells
R
U5
Ψ
PBS
Peptide
IRES
Neo
PPT
U3
R
Integrated vector DNA in target cells
U3
R
U5
Ψ
PBS
Peptide
IRES
Neo
PPT
U3
R
U5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00231

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 15/10, C12N 15/86, C12Q 1/68, G01N 33/68
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EPOQUE, MEDLINE, BIOSIS, DERWENT BIOTECH ABSTRACTS, CA, US PATFULL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIO/TECHNOLOGY, Volume 12, October 1994, Marta Duenas et al, "Clonal Selection and Amplification of Phage Displayed Antibodies by Linking Antigen Recognition and Phage Replication" page 999	1-26
X	WO 9504824 A1 (MEDVET SCIENCE PTY. LTD.), 16 February 1995 (16.02.95), page 7, line 10 - line 27, figure 1	1-26

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
16 Sept 1996

Date of mailing of the international search report
17 -09- 1996

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00231

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Dialog Information Services, file 154, MEDLINE, Dialog accession no. 08151796, Medline accession no. 92289796, Schumacher TN et al: "Synthetic peptide libraries in the determination of T cell epitopes and peptide binding specificity of class I molecules"; & Eur J Immunol (GERMANY) Jun 1992, 22 (6) p1405-12	1-26

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00231

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-26
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The wording "phenotypic trait" in claim 1 is to vague to permit an adequate search, hence has the search been limited to the examples.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

05/09/96

International application No.
PCT/DK 96/00231

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9504824	16/02/95	AU-A- 7342394	28/02/95
		AU-D- PM352094	00/00/00

Form PCT/ISA/210 (patent family annex) (July 1992)